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Abstract: Influenza A viruses circulating in humans from 1950 to 1987 featured a nonstructural (NS1) protein with a C-terminal extension of seven amino acids. The biological significance of this NS1 elongation remained elusive. We observed that replication kinetics of the wild-type virus A/Hong Kong/01/68 (H3N2) and a mutant encoding a truncated NS1 were indistinguishable in most experimental systems. However, wild-type virus outcompeted the mutant during mixed infections, suggesting that the NS1 extension conferred minor growth advantages.

DOI: https://doi.org/10.1128/JVI.01271-13

Emergence of a C-terminal seven amino acid elongation of NS1 around 1950 conferred a minor growth advantage to former seasonal influenza A viruses

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Running title: NS1 of A/Hong Kong/01/68

Keywords: Orthomyxoviruses, Influenza A virus, NS1.

Word count abstract:, 73 ; main text: 1472

Number of figures: 3, one color

Abbreviations: ffu, focus-forming units; HK68, A/HK/01/68; IFN, interferon; moi, multiplicity of infection; Mx1, Myxovirus resistance gene; NLS, nuclear translocation signal, NS1(del7), non-structural protein NS1 with a deletion of its 7 C-terminal amino acids; pfu, plaque-forming units;

Summary:

Influenza A viruses circulating in humans from ~1950 to ~1987 featured a non-structural (NS1) protein with a C-terminal extension of seven amino acids. The biological significance of this NS1 elongation remained elusive. We observed that replication kinetics of wild-type A/Hong Kong/01/68 (H3N2) and a mutant encoding a truncated NS1 were indistinguishable in most experimental systems. However, wild-type virus out-competed the mutant during mixed infections, suggesting that the NS1 extension conferred minor growth advantages.

Main Text

The non-structural NS1 protein of influenza A viruses is an important virulence factor (17). Viruses with NS1 deletions are attenuated in interferon (IFN)-responsive systems, indicating that NS1 counteracts the IFN-induced antiviral host defence (6, 11). The anti-IFN activity of NS1 affects IFN induction as well as the action of IFNs and IFN-induced genes (ISGs) (10). The NS segment of seasonal human influenza A viruses usually codes for an NS1 of 230 amino acids consisting of an N-terminal RNA-binding domain (amino acids 1-73) and a C-terminal effector domain (74-230) (Fig. 1A) (1). During the evolution of human influenza A viruses in the 20th century, the NS segment was preserved (18), but encountered a constant genetic drift (3, 13). Around the year 1950, the viruses gained a C-terminal elongation of seven amino acids, from 230 to 237, by a single nucleotide exchange in codon 231. This alteration replaced the stop codon (UGA) by an arginine codon (CGA) but did not affect the NS2 gene product. The NS1 elongation persisted in the progenies of the H2N2 pandemic virus of 1957 as well as in the H3N2 pandemic virus of 1968, but got successively lost again around 1987 (15). The significance of this elongation for viral replication is presently unknown.

We rescued the prototype virus of the 1968 pandemic encoding NS1 of 237 amino acids A/Hong Kong/01/68 (H3N2) (2), called here HK68(wt), from cDNA as described (8). Mutant virus HK68(del7) encoding NS1 of 230 amino acids was generated by introducing a single C to U nucleotide exchange in codon 231 that does not alter NEP/NS2. Wild-type and mutant viruses which were otherwise genetically identical grew to comparable titers in embryonated chicken eggs (not shown) and showed comparable growth kinetics in MDCK cells (Fig. 1B).

A deletion of the C-terminal seven amino acids in NS1 of strain A/Udorn/72 reportedly affected its second nuclear translocation signal (NLS2) at position 219 to 237 (15). Therefore, we analyzed the intracellular localization of NS1 in human alveolar epithelial A549 cells infected with HK68(wt) or HK68(del7) by immunofluorescence as described (20). Both versions of NS1 accumulated in the nuclei early in infection but outside of the nucleoli, similar to the viral nucleoprotein (NP) (Fig. 1C). At later time points, NP staining was also detectable in the cytoplasm whereas NS1 stayed mostly in the nucleus.

An important function of NS1 is the suppression of the IFN system of the host. To investigate whether the C-terminal extension of NS1 would influence this activity,

mouse embryo fibroblasts (MEF) were infected with HK68(wt) or HK68(del7). The cells were obtained from IFN-$\beta^{del\beta\text{-}luc/del\beta\text{-}luc}$ reporter mice expressing firefly luciferase under the control of the IFN-β promoter (12). Both viruses caused a 27-fold increase in reporter gene expression in comparison to uninfected cells (Fig. 1D). Comparable infection by the two viruses was monitored by Western blot for NS1 and NP. To compare the IFN-inducing capacity of the two viruses in human cells, we determined the type I IFN activity in acid treated supernatants of A549 cells infected for 24 h, using a bioassay (20). The mutant virus behaved like wild-type and did not induce measurable IFN activity above background. In contrast, a control virus carrying a mutation (R38A) in the RNA-binding motif of NS1 caused a 100-fold increase in IFN activity over mock, as expected (20) (Fig. 1E). Obviously, the addition of the seven C-terminal amino acids did not alter the IFN-suppressing properties of NS1 in cell culture.

To detect possible minor differences in the growth properties of HK68(wt) and HK68(del7), we performed sequential passages of mixtures of the two viruses. MDCK cells were infected with 0.001 moi of HK68(wt), HK68(del7) or a 1:1 mixture of both viruses. The progenies were subsequently passaged four times. Virus titers of each passage were comparable to those shown in figure 1B. RNA was isolated from the supernatants and used for segment-specific RT-PCR and sequencing. When the two viruses were passaged separately, sequence analysis did not detect any mutations that might have arisen during passage (Fig. 2A). The input material of the 1:1 mixture of the two viruses showed the presence of both codons at position 231. However, the percentage of HK68(wt) gradually increased during passaging and HK68(del7) disappeared in most cultures between the third and fourth passage (Fig. 2A and D), indicating a slight growth advantage for the virus with the extended NS1. HK68(del7) predominated only in one out of 10 independent passage experiments. Interestingly, when the passaging experiment was performed with a 1:4 mixture of HK68(wt) and HK68(del7), only HK68(del7) was present in the supernatants of four independent passaging experiments (Fig. 2D), indicating that the growth advantage of HK68(wt) is minimal.

To corroborate these findings in a more physiological context, we established differentiated human airway epithelial cell cultures (4) and infected these cells via the apical side of the air-liquid-interface with 0.01 moi of the two viruses. Both viruses grew to comparable titers (Fig. 2B). HK68(wt) dominated the virus population already

after the first passage in six out of eight independent 1:1 mixing experiments (Fig. 2C and D). In contrast, infection of a 1:9 mixture of HK68(wt) and HK68(del7) only yielded HK68(del7) even after 4 consecutive passages (Fig. 3D). Together, these experiments demonstrated that the advantage conferred by the seven amino acid extension of wild-type NS1 for growth in cell culture is detectable solely in competition experiments and suggest that the benefit is presumably minor.

To investigate the situation *in vivo*, IFN-$\beta^{del\beta\text{-}luc/del\beta\text{-}luc}$ reporter mice were infected intranasally with 5 x 10^4 pfu of either HK68(wt) or HK68(del7). In both cases, an approximately 22-fold increase in luciferase expression in the lungs was observed within 24 h (Fig. 3A). The viruses replicated in the lungs to similar titers of around 5 x 10^6 pfu/ml (Fig. 3B). We also determined viral growth in IFNAR1$^{0/0}$IL28R$\alpha^{0/0}$ mice that lack functional receptors for both type I and type III IFN (16). No significant differences in lung titers were observed 24h and 48h after infection with 1000 pfu (Fig. 3C). Next, we investigated Mx1-positive mice known to reveal even minor differences in the IFN-inducing capacity of viruses (11, 20). B6.A2G-Mx1 mice carrying intact alleles of the IFN-induced myxovirus resistance gene *Mx1* (19) were infected with 2000 pfu for 48 h. The growth of HK68(del7) was slightly but significantly reduced in these mice as compared to the wild-type virus (Fig. 3D), suggesting that the C-terminal elongation of NS1 may provide some benefit in the presence of a fully armed IFN system.

What is the molecular basis for the observed enhancement of HK68(wt) replication? Several possibilities need to be considered. The single G to A nucleotide exchange in the 5' region of the genomic vRNA might influence the packaging of segment 8. A minor role of the respective region in segment packaging has previously been suggested (5). On the other hand, the addition of seven amino acids might improve accessibility of NS1 for cellular interaction partners. The C-terminal part of NS1 is intrinsically disordered and provides a flexible tail for interaction partners (1, 9). Thus, a C-terminal, importin-α interacting motif NLS2(219-237) has been described for NS1 (7, 15). However, deletion of the last seven amino acids in NS1 of A/Udorn/72 did not completely disrupt importin-α binding, indicating a residual NLS2 function by the remaining positively charged amino acids (15). Accordingly, our immunofluorescence analysis of HK68-infected cells did not reveal changes in nuclear accumulation of

NS1(del7) compared to NS1(wt), suggesting that the N-terminal NLS1 and the truncated NLS2 are sufficient for proper NS1 nuclear translocation. A C-terminal histone mimicking motif (positions 226 to 229) has been described for NS1 of H3N2 strains that is modified by and can interact with proteins involved in histone modification and transcriptional regulation, suggesting a new strategy how NS1 modulates cellular gene expression (14). However, a possible effect of the C-terminal elongation of NS1 on the properties of this histone-mimicry sequence was not evaluated.

In summary, the present data indicate that the C-terminal elongation of NS1 proteins acquired by seasonal influenza A viruses around 1950 provided a small gain of fitness. The slightly enhanced growth of wild-type HK68 in Mx1-positive mice might argue for a better control of the innate antiviral defense by the virus. However, the beneficial effect is minor and may explain why the elongation got lost about 40 years later.

Acknowledgments

We thank Simone Gruber and Valentina Wagner for excellent technical assistance, Mikhail Matrosovich, Institute of Virology, Marburg, Germany, for providing the original human isolate of pandemic A/HK/01/68 and Robert G. Webster, St. Jude, Memphis, for providing the pHW2000 rescue vector.

Parts of this work were supported by a grant from the Deutsche Forschungsgemeinschaft (Ko 1579/5-2) to GK and by the Wissenschaftliche Gesellschaft in Freiburg to GK.

This work was conducted by Florens Lohrmann in partial fulfillment of the requirements for a M.D. degree from the Medical Faculty of the University of Freiburg, Germany.

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FIGURE LEGENDS:

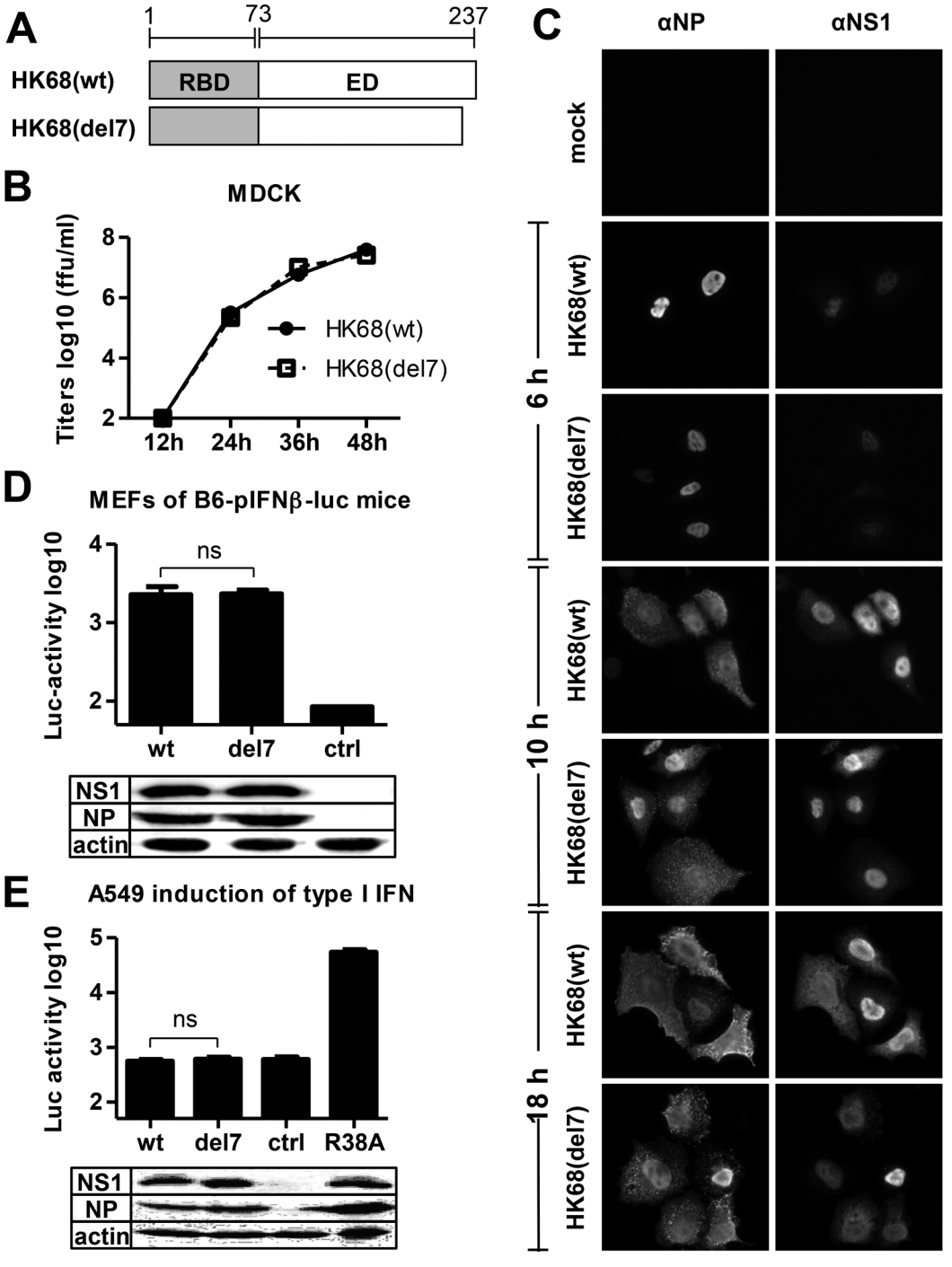
Figure 1: Growth and IFN-inducing capacity of wild-type and mutant HK68 viruses in cell culture. (A) Schematic representation of the functional domains of NS1. The NS1 protein of wild-type A/HK/01/68, HK68(wt), consists of 237 amino acids. HK68(del7) has a single nucleotide exchange at position 717 of segment 8 installing a stop codon in NS1 after 230 amino acids. RBD – RNA-binding domain, ED – effector domain. (**B**) Virus growth in cell culture. MDCK cells were infected in triplicates with 0.001 moi in the presence of 0.5 µg/ml of trypsin. At the indicated time points, supernatants were harvested and virus titers were determined. Error bars, SEM, were calculated from three independent experiments (differences were non-significant at all time points tested). (**C**) Subcellular localization of NS1(wt) and NS1(del7) as well as viral NP in infected A549 cells. Cells were infected with a moi of 0.5 for various time periods, fixed and stained with antibodies directed against NS1 and NP. Representative images out of three independent experiments are shown. (**D**) Activation of the IFN-β promoter (pIFNβ) in MEF. MEF expressing firefly luciferase (Luc) under the control of the IFNβ promoter were infected for 24 h with 0.5 moi of HK68(wt) and HK68(del7) or were mock treated (ctrl). Luciferase activity was determined in the MEF lysates. (**E**) Detection of type I IFN in the supernatants of A549 cells infected with 0.5 moi for 24 h. A known IFN-inducing virus encoding NS1(R38A) was used as positive control. Cell supernatants were treated with low pH to inactivate the virus and type I IFN was determined using 293 reporter cells expressing luciferase under the control of the Mx1 promoter (20). Data from three independent experiments were analysed using t-test (ns = differences are not significant). Viral NS1, NP and cellular actin in the whole cell lysates were detected by Western blot.

Figure 2: Wild-type HK68 out-competes the NS1 mutant virus during serial passage in cell culture. (**A**) Passage of HK68 on MDCK cells. Cells were infected with 0.001 moi of either HK68(wt), HK68(del7) or a 1:1 mixture of both viruses. Four serial passages were performed with a moi of 0.001. Viral RNA was isolated from cell culture supernatants between 48 and 60 h post infection, analyzed by segment 8-specific RT-PCR and sequenced. Codon 231 in negative sense orientation (TCG) for

HK68(wt) or (TCA) for HK(del7) is indicated. The results are shown for one representative experiment out of ten independent replicates of the 1:1 passaging experiments. (**B** and **C**) Competition experiments on human airway epithelium (hAEC) cells. Cells prepared from material of one donor were seeded in a 24 well format and were infected with 0.01 moi of the two viruses or a 1:1 or 1:9 mixture of HK68(wt) and HK68(del7). Supernatants were collected from the apical surface after the indicated time points and virus titers were determined (B). Error bars, SEM, were calculated from three independent experiments. (C) Viral RNA was isolated from the supernatants at 48 h post infection and sequences were determined. Codon 231 in the negative sense orientation is shown for one representative experiment out of eight independent experiments. (**D**) Summary of the sequence analysis for the individual competition growth experiments in MDCK and hAEC cells. The numbers indicate the frequency of detection of NS1(wt) versus NS1(del7) in all independent mixing experiments. Data are shown for the fourth MDCK passage of the 1:1 and 1:4 mixtures, and for the first passage of the 1:1 mixture and the fourth passage of the 1:9 mixture in hAEC.

Figure 3: IFN-inducing capacity and growth of wild-type and mutant HK68 *in vivo*. Groups of C57BL/6 (B6) reporter mice (n = 9) expressing firefly luciferase under the control of the IFN-β promoter (pIFNβ) were infected intranasally with $5x10^4$ pfu of HK68(wt) and HK68(del7) or were mock treated (ctrl). At 24 h p.i. luciferase activity (Luc) (**A**) and virus titers (**B**) were determined in the lung homogenates. (**C** and **D**) Infection of IFN-nonresponsive or fully competent mice. Lung titers of HK68(wt) and HK68(del7) in B6-IFNAR/IL28R double knock-out mice (n = 4-5) (C) and B6.A2G-$Mx1^{+/+}$ mice (n = 8) (D) were determined after intranasal infection with 1,000 and 2,000 pfu, respectively (ns = not significant, $^{*}p = 0.0286$).

Figure 1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HK68(wt)
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NP
actin
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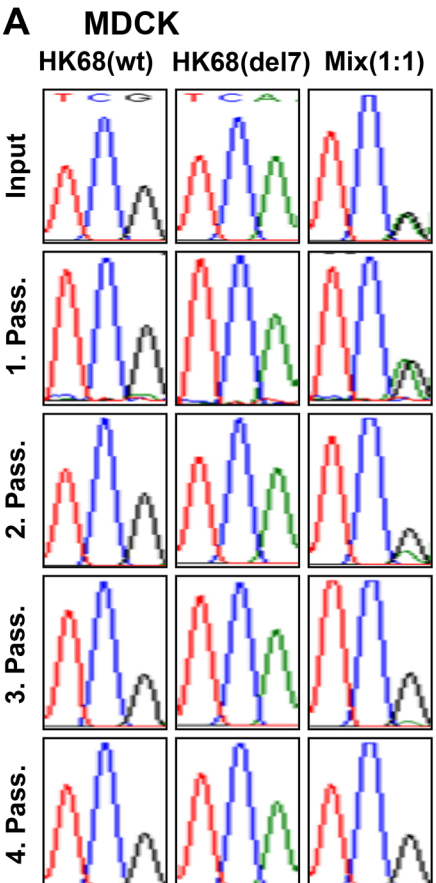
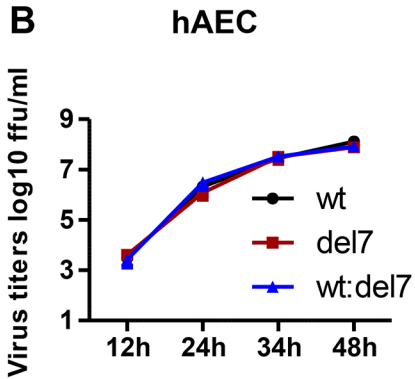
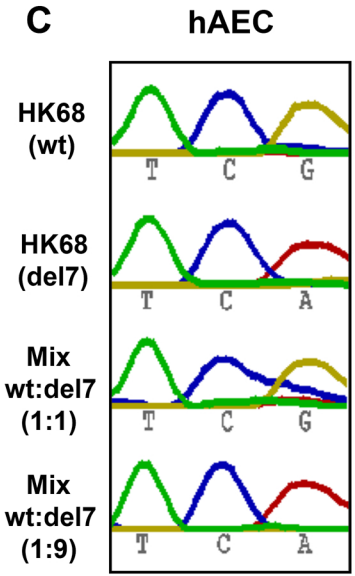
Figure 2



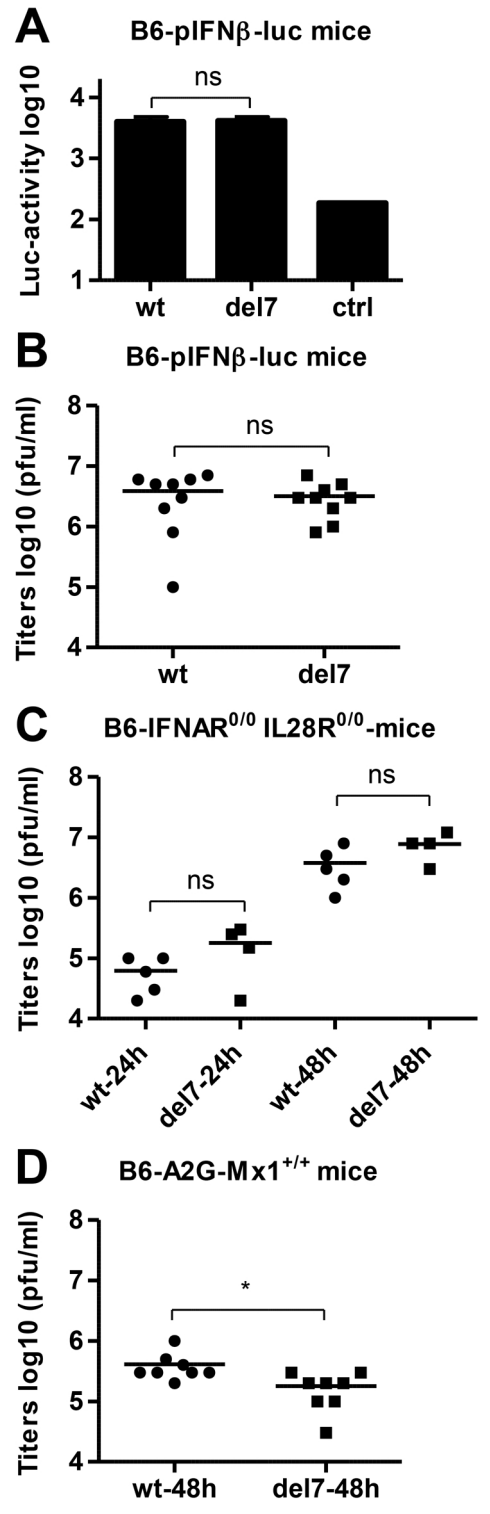




D

	MDCK wt:del7 (1:1)	MDCK wt:del7 (1:4)	hAEC wt:del7 (1:1)	hAEC wt:del7 (1:9)
wt>>del7	9/10	0/4	6/8	0/3
wt=del7	0/10	0/4	1/8	0/3
wt<<del7	1/10	4/4	1/8	3/3

Figure 3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B6-IFNAR0/0 IL28R0/0-mice
Titers log10 (pfu/ml)
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wt-24h
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